

C2 -- Any non-specificity can be avoided by fine-tuning the reaction conditions such as by adjusting the annealing temperature and the reaction temperature during amplification, and/or adjusting the length and G/C content of the primers. These adjustments are routinely done in the standard PCR amplification protocol. In short, although the partly-fixed primers have a random sequence component, a sub-population of the primer molecules will have the exact sequence that would bind with the exact target sequence. The proportion of the molecules with exact sequence that would bind with the exact target sequence will vary depending on the number of random characters in the partly-fixed primers. For example, a primer 11 nucleotides long with 6 characters fixed and 5 characters random, one in about 1000 primer molecules will have the exact sequence complementary to the target sequence on the template.

By increasing the concentration of the primers appropriately, a comfortable level of PCR amplification required for sequencing can be achieved. When primer concentration is increased, it requires an increase in the concentration of magnesium, which is required for the function of the polymerase enzyme. The excess primers (and "primer-dimers" formed due to excess of primers) can be removed after amplification reaction by a gel-purification step.

Any non-specific binding by any population of the primers to non-target sequences can be avoided by adjusting (increasing) the temperature of re-annealing appropriately during DNA amplification. It is well known that the change of even one nucleotide due to point-mutation in some cancer genes can be detected by DNA-hybridization. Hybridization is routinely used for diagnosing particular cancer genes (*e.g.*, John Lyons, "Analysis of *ras* gene point mutations by PCR and oligonucleotide hybridization," in PCR Protocols: A guide to methods and applications, edited by Michael A Innis et al., (1990), Academic Press, New York). This is done by adjusting the "re-annealing" or "melting-temperature", and fine-tuning the reaction conditions. Thus the binding of non-specific sequences even with just one nucleotide difference compared to the target binding-site in the template sequence can be avoided.

The minimum length of primer for highly specific amplification between primers on a template DNA is usually considered to be about 15 nucleotides. However, in the present invention, this length can be reduced by increasing the G/C content of the primers to 12-14 nucleotides. --